1094-Plat
Labeling Freedom for the Single Molecule Microscopist
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Observation of single molecule fluorescence has matured into a central tool to study biomolecular structure and dynamics. As hardware and data analysis technology dramatically improved over the last decade, site-specific labeling of proteins with small but highly photostable fluorescent dyes has turned into a major bottleneck for biological applications. In vitro, traditional approaches to label natural amino acids are the most widely applied, but due to the high abundance of even rare cysteines in larger biological machineries, few protein systems are accessible. Still more difficult to achieve is the ultimate goal of visualizing protein structure and dynamics in living cells and organisms. Compared to small organic fluorophores, however, fluorescent proteins, such as GFPs, are bigger and typically have worse photophysical properties, but since they can be genetically attached to any protein, they are the usual choice for in vivo studies. We have now developed a semi-synthetic strategy based on a novel artificial amino acid that is easily and site-specifically introduced into any protein by the natural machinery of the living cell. Expressed proteins only differ from their natural counterparts by very few atoms, constituting a ring-strained cyclooctyne functional group. We show that this completely inert and non-toxic group can be stably incorporated into any protein and readily reacts with commercially available single molecule fluorophores without the need of special reagents, catalyst or non-physiological buffer conditions. Similarly to fluorescent proteins, the dye attachment site is genetically encoded and will thus facilitate precise labeling of proteins in vivo by only changing a single amino acid. In fact, the speed and specificity of this method holds great promise for applications of single molecule and super resolution techniques in living cells, and new experimental results demonstrating this potential will be presented.

1095-Plat
Computational Predictions of Exponential and Non-Exponential Tryptophan Fluorescence Decay in NATA, the Villin Headpiece Subdomain, and other Proteins
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Decay of fluorescence from a single Tryptophan (Trp) in a peptide or protein is sometimes exponential and sometimes non-exponential. Understanding the details of this behavior has proven elusive. We have had qualitative success predicting average fluorescence quantum yields for a variety of proteins based on the stabilization of the charge transfer (CT) state arising from electron transfer from the aromatic ring to a backbone amide by the protein electrostatic environment. Here we report 100ns - 1 microsecond MD simulations, augmented by quantum mechanical (ZINDO) computations of the fluorescing and CT states, on N-acetyl-tryptophan-amide (NATA) and 20 single Trp proteins, including the highly studied fast folding villin headpiece. Although the environment for NATA in water is very similar to that of solvent exposed Trps on the surface of a protein, which almost always exhibit non-exponential decay, NATA shows a surprisingly pure single-exponential decay. In our simulations, all possible rotamer states are well represented, and transitions between rotamers happen at a rate of about 1 per 5 ns. Preliminary results indicate that rare conformations in which an amide carbonyl is H-bonded by 2-3 waters produce spikes of high quenching, more or less independent of rotamer state. Survival curve averaging of the 600 ns trajectory yields a single exponential decay of near 3 ns. For folded villin at 300 K, we find that rotamer transitions on Trp occur only every ~100 ns and that quenching by the nearby His+ happens only during these transient events, although the helix is always intact. Villin is therefore predicted to show extreme heterogeneity in lifetimes. This provides a mechanism for Trp fluorescence to report the global folding rate. Heterogeneity for the other proteins will also be discussed.

1096-Plat
Kinetics of Biotin Derivatives Binding to Avidin and Streptavidin
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The on-rate constants (k_{on}) of biotin (B) binding to avidin (AV) and streptavidin (SAV) are believed to be diffusion limited (10^9 $M^{-1}s^{-1}$). In this study; we asked whether these reactions were actual diffusion controlled, what association model and thermodynamic cycle describe the process, and what are the functional differences between AV and SAV. We have studied the B association by two stopped flow methodologies that used: I) fluorescent probes attached to B; and II) unlabeled B and HABA [2-(4'-hydroxyazobenzene)-benzoic acid]. The reactions were carried out at several temperatures, pH's and under pseudo first-order conditions with: oregon green biocytin (BcO), biotin-4-fluorescein (BFl), biotin-DNA duplex, and unlabeled B. We obtained the spectroscopy properties of the bound dye-biotin complexes to have an insight of the chemical environment surrounding B. The association data showed not cooperativity between the 1st and the 4th binding sites of AV. The k_{on} values of SAV were faster than AV's, but in both cases were slower than those expected for a diffusion limited reaction. Furthermore, the Arrhenius plots revealed strong temperature dependence with large activation energies (6-15 kcal/mol) that did not correspond either to a diffusion limited process (3-4 kcal/mol). The outcomes indicated that *AV binding sites were deeper and less accessibility than SAV*. In addition, we are reporting, *for the first time*, a second order displacement rate constant of a bound SAV complex when challenged with free B; results that are relevant for the purification technology base on these proteins. Finally, we propose a simple reaction model with a single transition state whose forward energetic parameters complete the thermodynamic cycle in excellent agreement with previous studies.

1097-Plat
Nonequilibrium Molecular Dynamics of Trp Zwitterion in Water: Picosecond Fluorescence Measurements Versus Computer Simulations
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This is an experimental test of MD simulations on the picosecond timescale. Tryptophan zwitterion in TIP3P water at 278°K was simulated using CHARMM22 forcefield with the excited-state Trp atomic charges from [Toptygin et al., *J. Phys. Chem. B* **2010,** *114,* 11323]. Six stable excited-state rotamers of Trp sidechain were found with the population density peaks near (χ_1,χ_2) = (67°,80°), (−170°,57°), (−65°,115°), (65°,-85°), (−165°,-112°), (−65°,-80°). Curved boundaries between the rotamers on the (χ_1,χ_2) map were drawn along the troughs of the population density. Population density distribution within the boundaries of one rotamer reaches equilibrium in less than 20ps; equilibration between different rotamers takes much longer. At t>20ps rotamer populations can be described by a system of six first-order homogeneous linear differential equations. The solution is a sum of six terms $V_{mn}\exp(-t/\tau_n)$. Population decay of each rotamer is not monoexponential and τ_n is not a lifetime. The same set of τ_n applies to all rotamers, but a different set of V_{mn} corresponds to each rotamer. The rotamers have slightly different fluorescence emission spectra, therefore fluorescence intensity is a sum of six terms $\alpha_n(\nu)\exp(-t/\tau_n)$, where α_n vary with the photon energy hν. We have determined τ_n and $\alpha_n(\nu)$ in the global analysis of spectrally- and time-resolved fluorescence data (time resolution 65ps FWHM). Only four exponential terms could be resolved from the experimental data in H_2O at 5°C (τ_1 = 4780ps, τ_2 = 2500ps, τ_3 = 867ps, τ_4 = 411ps); according to MD simulations the fifth term (τ_5 = 241ps) has a very small amplitude, and the sixth (τ_6 = 22ps) is faster than the time resolution. For a precise agreement between the experimental and simulated values of τ_n it is necessary to lower all potential barriers between rotamers by 0.178kcal/mol. This shows that fluorescence spectroscopy can be used to fine-tune torsional parameters.

Platform: Protein Folding & Stability

1098-Plat
Measurement of Average Transition-Path Time for Protein Folding in Single Molecule FRET Experiments
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The transition-path is the tiny fraction of an equilibrium molecular trajectory when a transition occurs between two states, and appears as an instantaneous jump in the measured signal in single molecule force or fluorescence experiments. Transition-paths are readily observed in atomistic molecular dynamics simulations for systems with fast kinetics, but have never been observed experimentally for any system in the condensed phase. The importance of the transition-path in protein folding is that it contains all the mechanistic information on how a protein folds and unfolds and is predicted from both theory and simulations to be heterogeneous. As a first step toward observing transition-paths in protein folding, we previously estimated an upper bound of ~200 microseconds for the transition-path time of protein G using single molecule FRET spectroscopy, 10,000 times shorter than the average unfolded-state waiting-time of ~2 seconds (Chung et al., PNAS 2009). The biggest obstacle to resolving a transition-path is to detect a sufficient number of photons during a single transition-path. To overcome this problem, we employed

a fully-automated data acquisition system to collect a very large number of photon trajectories at high illumination intensities, and carried out a collective photon-by-photon analysis of the transitions between the folded and unfolded states using a maximum likelihood method (Chung et al., JPC A 2011). We determined a transition-path time of ~2 microseconds for a WW domain that folds in ~100 microseconds and an upper bound of ~15 microseconds for protein GB1 that folds in ~2 seconds. The transition-path times for the two proteins differ by less than 10-fold while the folding rates differ by a factor of 20,000. This result shows that a slow-folding protein can fold almost as fast as a fast-folding protein when folding actually occurs!

1099-Plat
Influence of Calcium Binding on the Folding Properties of Single Calmodulin Molecules Observed with Optical Tweezers
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Calcium sensing and the control of signaling pathways play important roles in the control of cellular processes, ranging from immune response to apoptosis. The calcium-dependent signal transducer calmodulin is one of the key players involved. Relatively unstable at low calcium concentrations, calmodulin is greatly stabilized upon calcium binding, undergoes a conformational change and is able to bind other proteins for a specific response.
We use a dual beam high resolution optical tweezers setup to investigate the folding/unfolding properties of single calmodulin molecules. Using force both as a denaturant and a reporter for molecular extension we can tune the equilibrium between folded, intermediate, and unfolded states. Already in relatively small multi-domain proteins such as calmodulin, complex networks with on and off-pathway states can be found. We investigate the equilibrium fluctuations of calmodulin over several minutes and directly observe of the effects of calcium binding on the folding process.

1100-Plat
Cavities in the Hydrophobic Core Govern Pressure Unfolding of Proteins
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Since Bridgman's seminal experiments on high-pressure denaturation of albumen in 1914[1], the origin of pressure unfolding of proteins has remained unresolved. We report here a systematic study of the contribution of cavities to the volume difference between unfolded and folded states (ΔV_u), using 10 single point variants of staphylococcus nuclease (SNase). Each mutation is localised in a strategic position and was designed to change a large buried hydrophobic side chain into alanine, thus opening tunable cavities in the SNase structure. For every variant, a crystal structure confirmed the presence of the designed cavity with no detectable presence of water molecules. High-pressure fluorescence experiments show significantly larger ΔV_u values for the cavity mutants in comparison to the reference protein. This demonstrates that solvent-excluded cavities make a major contribution to ΔV_u. Thus, pressure effects have their origin in a property of the folded states of proteins, unlike temperature and chemical denaturants, whose effects are governed by exposed surface area in the unfolded state. High-pressure NMR experiments on 4 cavity mutants, recording HSQCs peak intensities up to 2500 bar, allowed precise estimations of the apparent ΔV_u monitored by more than two-thirds of the residues. An innovative combination of the site-specific NMR data and Go-model simulations revealed significant departures from the apparent two-state folding process for the SNase reference protein and the cavity mutants. This study opens up highly promising perspectives on the use of high pressure for characterization of folding landscapes inaccessible by other methods.
1. Bridgman, P. W. *J. Biol. Chem.* 1914.19, 511–512.

1101-Plat
The Fast and the Slow: Folding and Trapping of λ6-85
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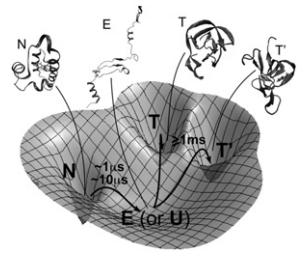
Molecular dynamics simulations that combine many μs trajectories have recently predicted that a very fast-folding protein like lambda repressor fragment $\lambda_{6\text{-}85}$ D14A could have a millisecond kinetic phase. We investigated this possibility by detecting temperature jump relaxation to 5 ms. While $\lambda_{6\text{-}85}$ D14A shows no significant slow phase, two even more stable mutants do. A slow phase of $\lambda_{6\text{-}85}$ D14A does appear in mild denaturant. The experimental data (and we believe also the computational modeling) is consistent with the following hypothesis: $\lambda_{6\text{-}85}$ takes only microseconds to reach its native state from an extensively unfolded state, while the latter takes milliseconds to reach compact traps containing beta sheet structure. $\lambda_{6\text{-}85}$ is not only thermodynamically, but also kinetically protected from reaching intramolecular analogs of beta sheet aggregates while folding.



1102-Plat
Membrane Protein Stabilities and M-Values
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Understanding and prediction of membrane protein structures requires knowing the physical forces stabilizing them. However, such measurements are rare for membrane proteins. The few measurements that have been made were carried out under very different experimental conditions using different lipid bilayer compositions and geometries, which makes derivation of sequence-structure-energy relationships difficult. We have overcome many technical obstacles to measuring folding free energies of membrane proteins and will present novel measurements of the free energy of unfolding and the m value for several membrane proteins from E. coli. These stability measurements were accomplished in the same lipid bilayer system, and our results indicate that the trends in the stability of those proteins can be explained by the degree of hydrophobicity of their lipid-facing residues in their transmembrane regions. We also found that the sensitivity of these membrane proteins to chemical denaturation (as judged by their m values) was consistent with the sensitivity of water-soluble proteins having comparable differences in the solvent-exposure between their folded and unfolded states.

1103-Plat
Quantifying the Dimerization Energy of a CLC Transporter in Lipid Bilayers
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Recently, a structurally stable and functional monomeric form of the normally homodimeric Cl^-/H^+ antiporter CLC-ec1 was designed by introducing two tryptophan mutations at the dimer interface, I201W and I422W. Several single tryptophan mutant constructs show intermediate stability between monomer and dimer state, as observed by size exclusion chromatography. In addition, the monomer and dimer populations can be shifted by adding lipids to the purified protein in detergent micelles, indicating that the system is undergoing reversible dimerization. We are now developing CLC-ec1 into a model for measuring dimerization energetics in a lipid bilayer. To measure the free energy, as well as enthalpic and entropic contributions, we must determine the fraction of monomer and dimer in the total protein population at different temperatures. To do this, we introduce cysteine residues into the extracellular loop adjacent to the dimerization interface, and fluorescently label the protein with tetramethylrhodamine- (TMR) or fluorescein-maleimide. The fluorescent protein is then reconstituted into liposomes at known concentration, and the fraction of dimer is measured in one of two ways. In the first method, protein is labeled with TMR at a position that allows the rhodamine molecules to undergo self-quenching by forming non-fluorescent pairs when in the dimer state. Addition of 0.5% SDS dissociates the dimer, as confirmed by glutaraldehyde cross-linking, leading to an increase in fluorescence at the rhodamine peak emission wavelength, and a measurement of the dimer population. In the second method, protein is co-labeled with fluorescein and TMR, and the Förster resonance energy transfer signal is measured from the dimer complexes. These studies introduce CLC-ec1 reversible dimerization as a simplified model for the thermodynamics of membrane protein folding and TM helix recognition in the membrane environment.

1104-Plat
Cysteine Shotgun-Mass Spectrometry (CS-MS) Reveals Dynamic Sequences of Protein Structure Changes within Mutant and Stressed Cells
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Questions of if and when protein structures change within cells pervade biology and include questions of how the cytoskeleton sustains stresses exerted on or by cells _particularly in mutant versus normal cells. Cysteine shotgun labeling with fluorophores is analyzed here with mass spectrometry of the spectrin-actin membrane skeleton in sheared red blood cell ghosts from normal and diseased mice. Sheared samples are compared to static samples at 37 °C in terms of cell membrane intensity in fluorescence microscopy, separated protein fluorescence, and tryptic peptide modification in liquid chromatography-tandem mass spectrometry (LC-MS/MS). Spectrin labeling proves to be the most